SUPPLEMENTAL FIGURES AND LEGENDS

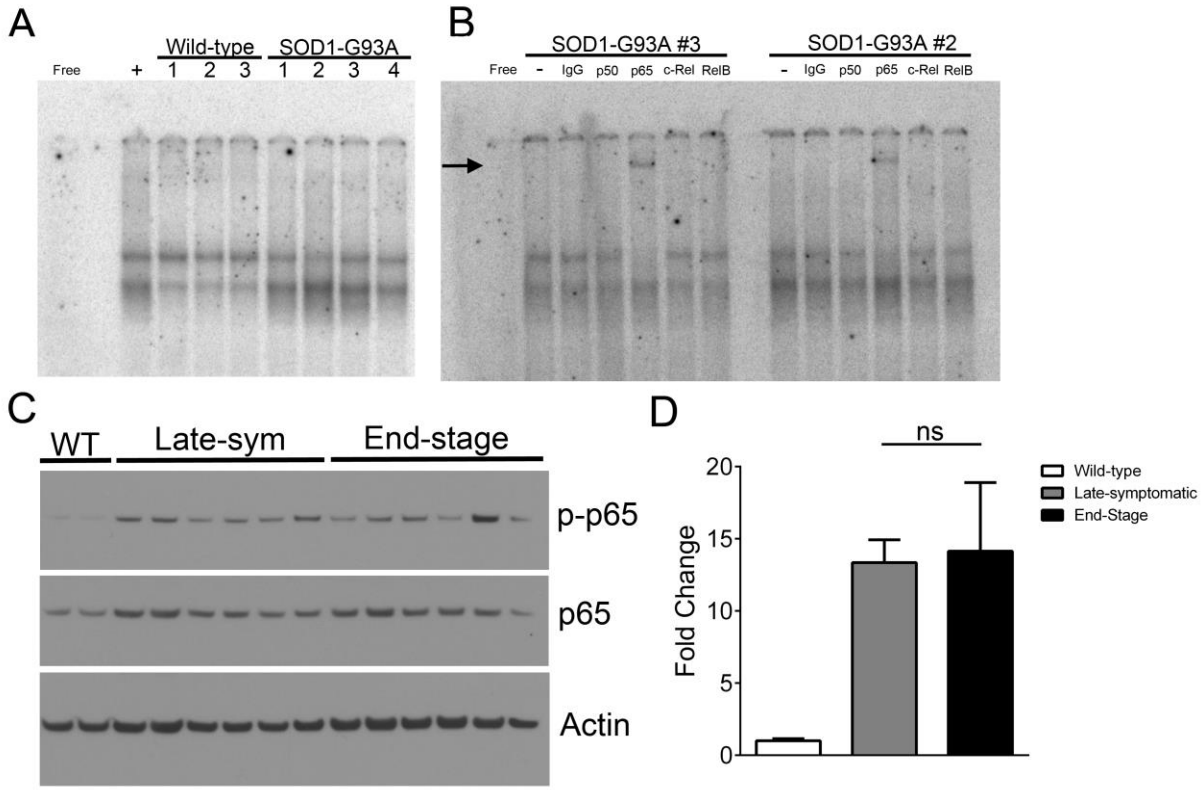

Figure S1. The classical NF-κB pathway is activated in SOD1-G93A mice, related to Figure 1.

(A) Electrophoretic mobility shift assay of total spinal cord nuclear extracts from 130 day old wild-type mice and end-stage SOD1-G93A mice. Free represents the probe free condition. "+" represents positive control from IKKβCA mice.

(B) Supershifts of nuclear extracts from SOD1-G93A sample #3 and #2. Arrow shows supershifted band from p65 antibody. "-" indicates no antibody is added.

(C) Immunoblot of lumbar spinal cord protein lysate from wild-type (n=2), late-stage (n=6), and end-stage (n=6) SOD1-G93A mice. The blot was probed for phospho-p65 and reprobed for total p65 (middle) and Actin (bottom) as loading controls.

(D) Fold change of the immunoblot in (C) determined using Image J to measure band intensities of phospho-p65 normalized to p65/Actin. Phospho-p65 is upregulated by 13.4 ± 1.6 fold compared to wild-type at the late-symptomatic stage and by 14.1 ± 4.8 fold at end stage.
Error bars represent s.e.m.

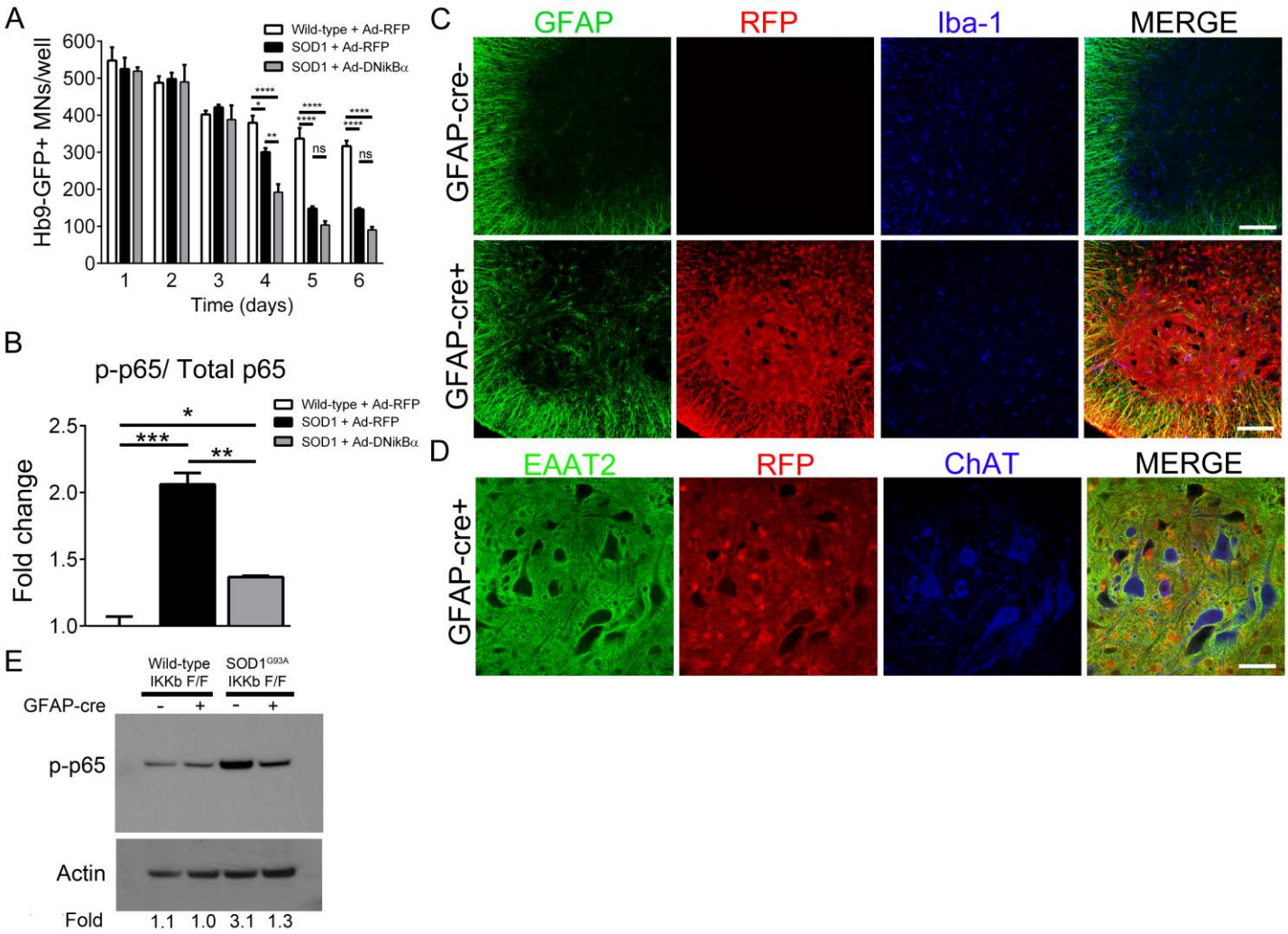

Figure S2. NF-κB inhibition in astrocytes does not confer neuroprotection *in vitro* or *in vivo* in the SOD1-G93A mouse model, related to Figure 1.

(A) Quantification of surviving Hb9-GFP+ motor neurons per well during 6-day co-culture with wild-type (white) or SOD1-G93A astrocytes infected with Ad-RFP (black) or Ad-IκBα-SR (gray). (n=3)

(B) Quantification of phospho-p65 by ELISA in wild-type and SOD1-G93A astrocytes infected by Ad-RFP or Ad-IκBα-SR and stimulated with 10ng/mL TNF-α for 12 hours.

(C and D) Representative images of GFAP-cre-negative and positive Rosa26-Stop^{Flox}-CAG-tdTomato mice. Native RFP fluorescence was analyzed for co-localization with immunohistochemical markers for (C) astrocytes (GFAP and EAAT2), microglia (Iba1), and (B) motor neurons (ChAT). Scale bar = 100 microns (top) 50 microns (bottom)

(E) Immunoblot of lumbar spinal cord protein isolated from WT; IKKβ$^{f/f}$; GFAP-cre-, WT; IKKβ$^{f/f}$; GFAP-cre+, and symptomatic SOD1-G93A; IKKβ$^{f/f}$; GFAP-cre-, SOD1-G93A; IKKβ$^{f/f}$; GFAP-cre+ mice probed for phospho-p65 (top) and Actin (bottom) confirm reduction in NF-κB activation in cre+ mice. Fold change represents band intensities of phospho-p65/Actin determined by ImageJ.
Error bars represent s.e.m. * , P< 0.05; **, P<0.01; **** , P< 0.0001

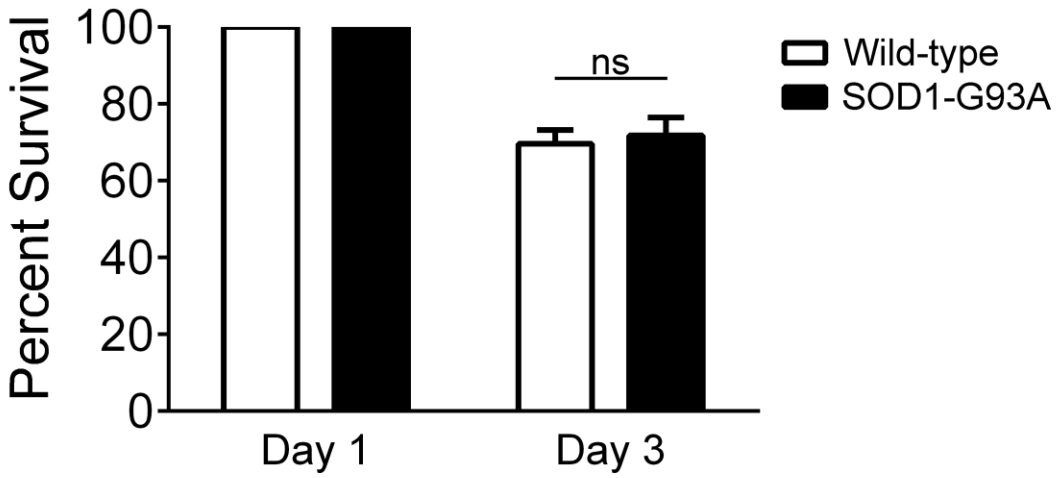

Figure S3. SOD1-G93A neonatal microglia are not toxic to Hb9-GFP+ motor neurons compared to wild-type microglia, related to Figure 4. Quantification of surviving Hb9-GFP+ motor neurons per well during co-culture with wild-type (white) or SOD1-G93A microglia (black). Error bars represent s.e.m.

Movie S1. Live-imaging of co-culture with Hb9-GFP+ motor neurons (green) and wild-type microglia (red), related to Figure 4.

Movie S2. Live-imaging of co-culture with Hb9-GFP+ motor neurons (green) and SOD1-G93A microglia (red), related to Figure 4.

Movie S3. Live-imaging of co-culture with Hb9-GFP+ motor neurons (green) and wild-type microglia overexpressing IκBα-SR (not fluorescently labeled), related to Figure 4.

Movie S4. Live-imaging of co-culture with Hb9-GFP+ motor neurons (green) and SOD1-G93A microglia overexpressing IκBα-SR (not fluorescently labeled), related to Figure 4.

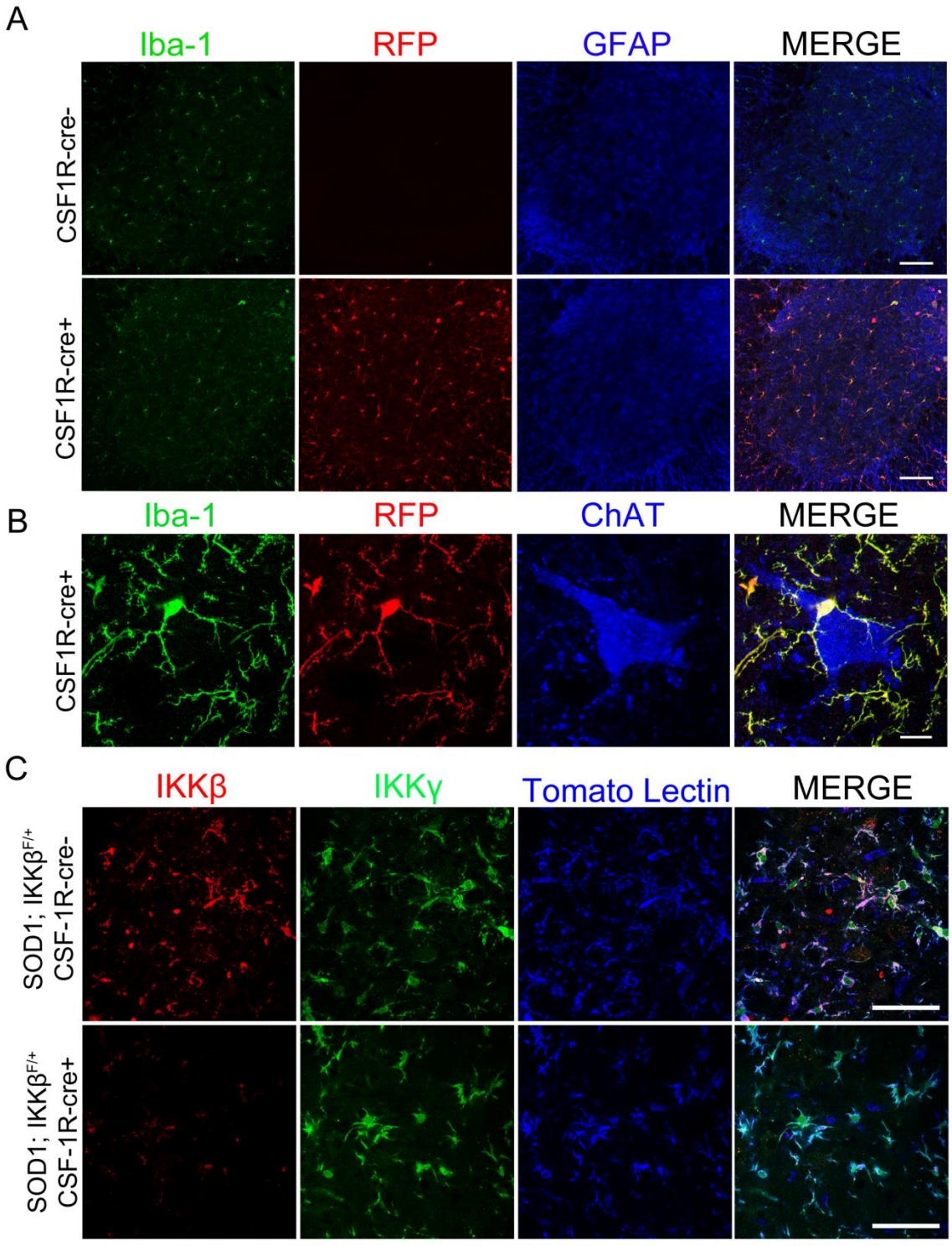

Figure S4. CSF-1R-cre is selectively expressed in microglia in the CNS, related to Figure 5.

(A and B) Representative images of CSF1R-cre-negative and positive Rosa26-Stop^{Flox}-CAG-tdTomato mice. Native RFP fluorescence was analyzed for co-localization with immunohistochemical markers for (A) microglia (Iba-1) and astrocytes (GFAP), and (B) motor neurons (ChAT). Scale bar= 100 microns (top) and 10 microns (bottom).

(C) Immunohistochemical analysis of end-stage SOD1-G93A; IKKβ$^{F/wt}$; CSF1R-cre negative and positive mice for IKKβ (red) and IKKγ (green) and tomato lectin (blue). Scale bar = 50 microns

Movie S5. Movie of representative SOD1-G93A; IKKβ$^{f/wt}$; CSF-1R-cre+ mouse compared to cre-negative littermate. Related to Figure 5.

Movie S6. Live-imaging of co-culture with Hb9-GFP+ motor neurons (green) and wild-type microglia expressing constitutively active IKKβ (not fluorescently labeled). Related to Figure 7.

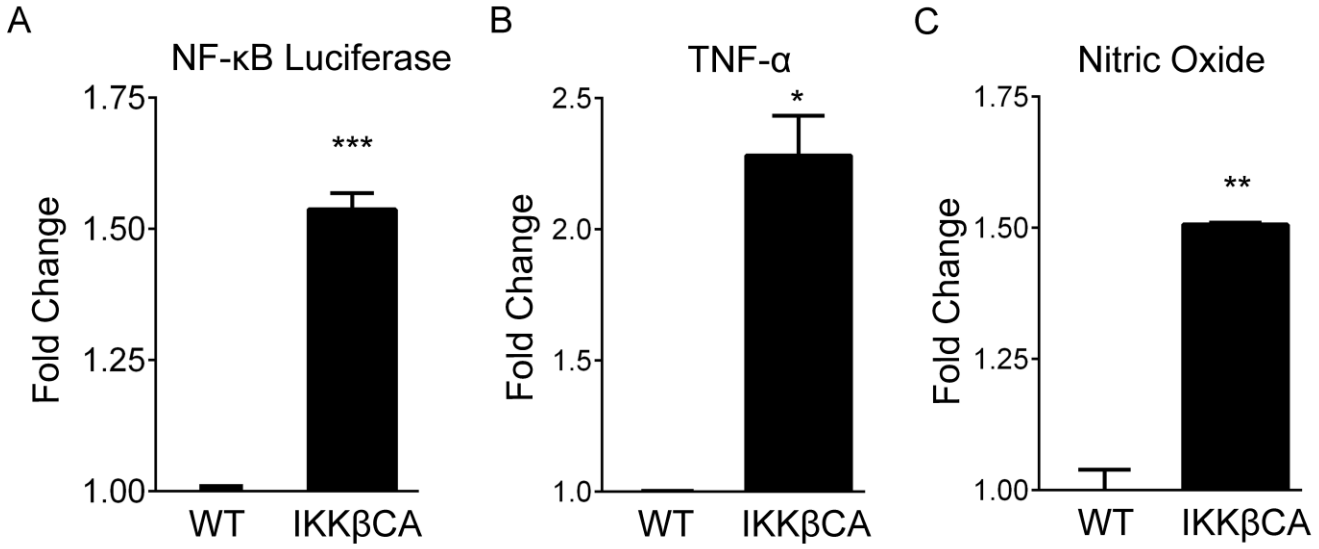

Figure S5. NF-κB activation in wild-type microglia *in vitro* induces microglial activation to a pro-inflammatory, neurotoxic phenotype, related to Figure 7.

(A) Luciferase assay of NF-κB activity in wild-type (white bar) and IKKβCA (black bar) microglia. Firefly luciferase was normalized to renilla luciferase.

(B and C) Quantification of TNF-α (B) and nitric oxide (C) in the co-culture medium by ELISA. Nitric oxide measured indirectly by sum of nitrate and nitrite.

Error bars represent s.e.m. * , P< 0.05, ** , P< 0.01

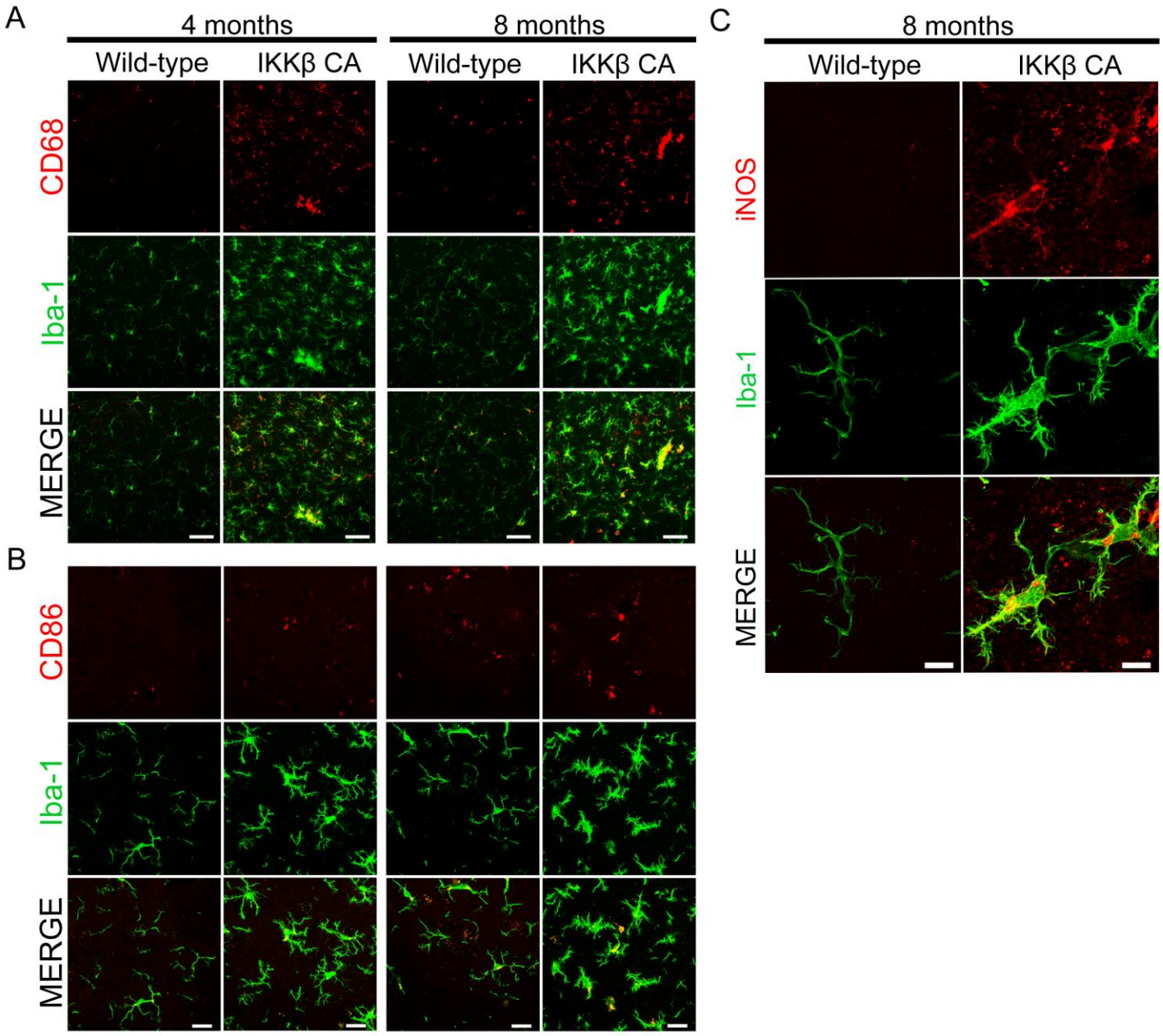

Figure S6. NF-κB activation in wild-type microglia *in vivo* induces microglial activation to a pro-inflammatory, neurotoxic phenotype, related to Figure 7.

(A) Immunohistochemistry of CD68 (red) and Iba1 (green) cells in lumbar spinal cord of WT and IKKβCA littermates at 4 and 8 months. Scale bar = 50 microns
(B) Immunohistochemistry of CD86 (red) and Iba1 (green) cells in lumbar spinal cord of WT and IKKβCA littermates at 4 and 8 months. Scale bar = 20 microns
(C) Immunohistochemistry of iNOS (red) and Iba1 (green) cells in lumbar spinal cord of WT and IKKβCA littermates at 8 months. Scale bar = 10 microns

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Supplemental Table 1. List of primers

Genotyping qualitative PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
human SOD1	CAT CAG CCC TAA TCC ATC TGA	CGC GAC TAA CAA TCA AAG TGA
Control for SOD1 reaction	CTA GGC CAC AGA ATT GAA AGA TCT	GTA GGT GGA AAT TCT AGC ATC ATC C
IKKβ	GTC ATT TCC ACA GCC CTG TGA	CCT TGT CCT ATA GAA GCA CAA
iCre	CAGGGCCTTCTCCACACCAGC	CTGGCTGTGAAGACCATC
Cre	GGACATGTTCAGGGATCGCCAGGCG	CGACGATGAAAGCATGTTTAGCTG
eGFP	GAG CTG AAG GGC ATC GAC TTC AAG	GGA CTG GGT GCT CAG GTA GTG G
negative for eGFP	TCAGGCCCACCTAGTCAGAT	AAAGCGGTCTGAGGAGGAA
tdTomato	CTG TTC CTG TAC GGC ATG G	GGC ATT AAA GCA GCG TAT CC
negative for tdTomato	AAG GGA GCT GCA GTG GAG TA	CCG AAA ATC TGT GGG AAG TC

Copy Number real time PCR

Primer	5' label	Sequence 5' --> 3'	3' Label
Transenic Probe	6-FAM	CTG CAT CTG GTT CTT GCA AAA CAC CA	Zen probe with Iowa Black
Internal Positive Control Forward	-	CAC GTG GGC TCC AGC ATT	-
Internal Positive Control Reverse	-	TCA CCA GTC ATT TCT GCC TTT G	-
hSOD1 Forward	-	GGG AAG CTG TTG TCC CAA G	-
hSOD1 Reverse	-	CAA GGG GAG GTA AAA GAG AGC	-
Internal Control Probe	Cy5	CCA ATG GTC GGG CAC TGC TCA A	Black Hole Quencher 2

Supplemental Table 2. List of antibodies

Western blot

Antibody	Dilution	Vendor
Phospho-p65	1:500	Cell Signaling
p65	1:500	Cell Signaling
Beta-Actin	1:1000	Cell Signaling
IKK-beta	1:125	Imgenex

Immunohistochemistry

Antibody	Dilution	Vendor
GFP	1:400	Abcam
Tomato Lectin	1:300	Vector Laboratories
GFAP	1:500	Abcam
Iba-1	1:400	Wako
CD68	1:100	AbDserotec
CD86	1:100	Millipore
iNOS	1:100	Sigma
IKK-gamma	1:100	Cell Signaling
IKK-beta	1:100	Imgenex

Immunocytochemistry

Antibody	Dilution	Vendor
CD11b	1:200	AbDserotec
F4/80	1:100	AbDserotec
NG2	1:200	Millipore
ChAT	1:100	Millipore
Iba-1	1:500	Wako
GFAP	1:200	Abcam

Flow Cytometry

Antibody	Dilution	Vendor
APC-CD11b	1:50	eBiosciences
PE-CD45	1:25	eBiosciences

EMSA supershift and nuclear western blots

p65	1:1000	Santa Cruz Biotechnology
p50	1:1000	Santa Cruz Biotechnology
c-Rel	1:1000	Santa Cruz Biotechnology
Rel-B	1:1000	Santa Cruz Biotechnology
IgG	1:1000	Santa Cruz Biotechnology

Transgenic mice

All procedures were performed in accordance with the NIH Guidelines on the care and use of vertebrate animals and approved by the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children's Hospital. Animals were housed under light:dark (12:12 h) cycle and provided with food and water *ad libitum*. Transgenic female B6SJ/L(SOD1-G93A)1Gur/J mice and non-transgenic

littermates (Jackson Laboratories) were utilized for time course immunoblot studies and primary cell isolations. Transgenic male B6SJ/L(SOD1-G93A)1Gur/J mice were used for breeding with other transgenic lines. SOD1 transgene copy number was confirmed by real time PCR. SOD1-G93A-NFκB^{EGFP} reporter mice were generated by breeding SOD1-G93A mice to C57BL/6 NFκB^{EGFP} mice (Christian Jobin) (Magness et al., 2004). SOD1-G93A; hGFAP-cre; IKKβ$^{flox/flox}$ were generated by breeding SOD1-G93A mice to FVB hGFAP-cre (Jackson Labs) mice that had been crossed to C57BL/6 IKKβ$^{flox/flox}$ mice (Li et al., 2003). SOD1-G93A; CSF-1R-icre; IKKβ$^{flox/wt}$ were generated by breeding SOD1-G93A mice to C57BL/6 CSF-1R-cre mice (Deng et al., 2010) that had been bred to IKKβ$^{flox/flox}$ mice. CSF1R-cre; IKKβCA were generated my breeding CSF-1R cre mice to C57BL/6 Rosa26-Stop^{Flox}IKKβCA mice (Jackson Labs). Cre specificity was confirmed by crossing cre lines to C57BL/6 Rosa26-Stop^{Flox}-CAG-tdTomato (Jackson Labs) mice and assessed for tdTomato expression by immunohistochemistry. Genotypes were determined by qualitative PCR using the primers in Supplementary Table 1.

Disease scoring and behavior analysis

Mice were classified as “pre-symtomatic” when they displayed no clinical symptoms of disease and had not reached peak weight. “Onset” was determined at the stage mice reach peak body weight. The “symptomatic” stage was determined when mice had lost 10% of their body weight and displayed motor impairment tremors or impaired hindlimb splay reflex. The “late-symptomatic” stage was determined when mice experienced pronounced hindlimb paralysis, but could reach food and water using forelimbs. “End-stage” was determined when animals could no longer “right” themselves within 30 seconds after the animal was placed on its back.

Testing of motor function using a rotarod device (Columbus Instruments, Columbus, OH) began at 50 days of age. Each session consisted of three trials that were averaged on the elevated accelerating rotarod beginning at 5 r.p.m./minute measuring the time the mouse was able to remain on the rod. Grip strength measurements for hindlimb were tested weekly using a grip strength meter (Columbus Instruments). Each session consisted of three tests per animal and values were averaged.

Immunoblot analysis

Cells and tissues were homogenized in Tissue Protein Extraction Reagent (Pierce) with EDTA, Complete protease inhibitor (Roche) and Phospho-STOP (Roche). The samples were run on NuPAGE Novex 4-12% Bis-Tris polyacrilamide gels and transferred to a PVDF membrane (Life Technologies). Blots were blocked in 5% milk powder, 0.5% BSA in PBS-Tween for 1h, and then incubated for overnight at 4°C with primary antibody. Bound primary antibody was detected by horseradish peroxidase conjugated secondary antibody followed by chemiluminescence detection (ECL Western Blot Substrate, Pierce). Antibodies are listed in Supplementary Table 2.

Immunohistochemistry

Animals were deeply anesthetized with a lethal dose of Xylazene/Ketamine and perfused transcardially with saline, then 4% paraformaldehyde. Spinal cords were sectioned 40 μm thick using a vibrating blade microtome (Leica microsystems). Sections were incubated for 2h at room temperature in TBS+ 1% Triton-X + 10% donkey serum. Samples were incubated for 72h at 4°C with primary antibodies, followed by 2h incubation at RT with secondary antibodies. All images were captured on a Zeiss confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Antibodies are listed in Supplementary Table 2. For quantification of MNs and microglia, lumbar spinal cords were sectioned 40 μm thick from the end of thoracic level 14 to sacral level 1. For MN counts lumbar spinal cord sections were selected every 5th section from the first identifiable L1 section through L6 and sections were selected every 8th section for microglial quantification.

Electrophoretic mobility shift assays (EMSA) and nuclear western blots

EMSA and supershift analyses were performed on whole spinal cord nuclear lysates as previously described (Dahlman and Guttridge, 2012). Nuclear westerns were performed using the same nuclear lysates as used for the EMSAs. The antibodies against p65, p60, c-Rel, and RelB are listed in Supplementary Table 1.

Isolation and culture of adult primary astrocytes

Adult astrocyte cultures from brains of SOD1-G93A and wild-type littermates were prepared and purified as previously described (Miranda et al., 2012; Ray and Gage, 2006) with minor modifications.

Enzymatically dissociated cells were cultured for 2 to 3 weeks, and then shaken overnight when the cells reached confluency to eliminate contaminating microglia. Adhered confluent astrocytes were treated with cytosine arabinose (20 μM) for 48 hours to kill rapidly dividing cells and microglia. Astrocytes were cultured in DMEM GlutaMAX™ DMEM + 10% FBS + N2 + antibiotic-antimycotic (all from Life Technologies).

Isolation and culture of adult primary microglia

Adult microglia were isolated from brains of SOD1-G93A and WT littermates as previously described (Moussaud and Draheim, 2010) with minor modifications. 4-month old SOD1-G93A and WT littermate mice were deeply anesthetized and perfused transcardially with ice-cold Ringers solution (Fisher Scientific). Brains that appeared to not be fully exsanguinated were discarded. Brains were fragmented with a scalpel and incubated with an enzymatic solution containing papain for 60 minutes at 37°C, 5% CO2. The papain solution was quenched with 20% FBS in HBSS and centrifuged for 4 minutes at 200g. The pellet was resuspended in 2ml of 0.5 mg/ml DNase I (Worthington Biochemical) in HBSS and incubated for 5min at room temperature. The brain tissue was gently disrupted with fire-polished Pasteur pipettes and then filtered through a 70 micron cell strainer (Fisher Scientific) and centrifuged at 200g for 4 minutes. The resulting pellet was then resuspended in 20ml of 20% isotonic Percoll (GE healthcare) in HBSS. 20mL of pure HBSS was carefully laid on top the percoll layer and centrifugation was performed at 200g for 20 min with slow acceleration and no brake. The interphase layer containing myelin and cell debris was discarded, and the pellet containing the mixed glial cell population was washed once with HBSS and suspended in Dulbecco's modified Eagle's/F12 medium with GlutaMAX™ (DMEM/F12) supplemented with 10% heat inactivated FBS, antibiotic-antimycotic (all from Life Technologies) and 5 ng/ml of carrier-free murine recombinant granulocyte and macrophage colony stimulating factor (GM-CSF) (R&D systems). The cell suspension from four mouse brains were plated on a 15cm2 plate (Corning) coated with poly-l-lysine (Sigma) and maintained in culture at 37°C in a 95% air/ 5% CO2. The medium was replaced every 3 days until the cells reached confluency (after approximately 2 weeks). After the glial layer becomes confluent, microglia form a non-adherant, floating cell layer that can be collected, replated, and cultured for an extended period of time. After collecting the floating layer, microglia were incubated for 3 days without GM-CSF before re-plating for co-culture with MNs. Collected microglia were characterized by immunocytochemistry and flow cytometry (antibodies listed in Supplementary Table 1).

Direct isolation of microglia for western blot analysis was performed as previously described (Cardona et al., 2006; Henry et al., 2009).

Isolation and culture of primary neonatal microglia

Microglia cultures were prepared from 3-day old SOD1-G93A and wild-type littermate pups as previously described with some minor modifications (Xiao et al., 2007). After removing meninges, cortices were dissociated and digested with 0.1% trypsin at 37°C for 15 minutes. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Life Technologies) and DNase I (Worthington Biochemical) was added to the tissue homogenate. After centrifugation at 1200 RPM for 4 minutes, the cellular pellet was resuspended in DMEM with 10% FBS and plated 75cm^2 flasks. Medium was replaced 24 hours after plating. After 1 week incubation, the flasks were shaken overnight at 250 RPM at 37°C. After collection, the microglia were plated for co-culture with motor neurons at a density of 6,000 cells per 96-well as described in the "Microglia/MN co-culture" experimental procedures section.

MN differentiation

Mouse embryonic stem cells expressing GFP driven by the Hb9 promoter (HBG3 cells, kind gift from Tom Jessell) were cultured on primary mouse embryonic fibroblasts (Millipore) and differentiated to MNs with the addition of 2 μM retinoic acid (Sigma) and 2 μM purmorphamine (Calbiochem). After 5 days of differentiation, the embryoid bodies were dissociated and sorted for GFP on a FACSVantage/DiVa sorter (Becton Dickinson).

ELISAs

TNFα Quantikine ELISA kit (R&D Systems) was used according to manufacturer instructions to quantify the TNFα concentration in co-culture medium. Nitric oxide levels in the co-culture medium were determined using the Total Nitric Oxide and Nitrate/Nitrite Parameter Kit (R&D Systems) according to manufacturer instructions. Co-culture medium was collected, centrifuged for 2 minutes at 200g, and 50uL of medium was added to each well for analysis. Phospho-p65 and Total p65 ELISA kits were used according to manufacturer instructions to quantify NF-κB activation in cell lysates (Cell Signaling). All conditions were tested in triplicate.

Virus production

Transgenic SOD1 expression in microglia was knocked down by lentiviral transduction expressing short interfering RNA sequences previously described (Haidet-Phillips et al., 2011; Miller et al., 2006). Lentivirus SOD1-shRNA and scramble-shRNA were produced by transient transfection into HEK293 cells using calcium phosphate, followed by supernatant viral purification by ultracentrifugation. Adenoviral vectors (Ad-RFP, Ad-cre, and Ad-IκBα-SR) were purchase from Vector Biolabs. Microglia were infected with an MOI of 25 overnight, then washed with HBSS and incubated 3 days before co-culture with motor neurons.

AAV9-IκBα-SR injections

Adult tail vein injections were performed on 60 day old SOD1-G93A mice as previously described (Foust et al., 2008; 2010) with a 100 µl viral solution containing a mixture of PBS and 4×10^{12} DNase-resistant particles of scAAV9-CB- IκBα-SR (Virapur).

Flow cytometry of microglia cultures

Flow cytometric analysis of microglial cell surface markers was performed by first blocking Fc receptors with anti-CD16/CD32 antibody (eBiosciences, CA). Next, cells were incubated with anti-CD11b APC, anti-CD45 FITC (eBiosciences). Expression of these surface receptors was determined by flow cytometry using a Becton-Dickinson LSR II Cytometer. Ten thousand events were collected and microglia incubated with isotype control were used as a negative control. Flow data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Luciferase reporter assays

Primary microglia were plated at a density of 2×10^4 cells and transduced with 10 multiplicity of infection (MOI) of the inducible NFκB-responsive firefly luciferase reporter and 1 MOI of the cignal™ lenti renilla control using SureENTRY transduction reagent according to manufacturer manual (SABioscience). Adenoviral vectors encoding IκBα-SR and cre recombinase (Vector Biolabs) we used at an MOI of 25 to inhibit NF-κB. The cells were washed with PBS 24 hours post-infection. Firefly and renilla luciferase activities were

determined using the Dual-Glo Luciferase Assay System (Promega) 72 hours after transduction. The amount of firefly luciferase activity of the transduced cells was normalized to renilla luciferase activity.

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